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Biochimica et Biophysica Acta 1501 (2000) 25–32

BIOCHIMICA ET BIOPHYSICA ACTA

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Generation of rat eosinophils by recombinant rat interleukin-5 in vitro and in vivo

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Received 15 September 1999; received in revised form 17 December 1999; accepted 11 January 2000

Abstract

The addition of recombinant rat interleukin-5 (IL-5), which was purified from the hemolymph of silkworm *Bombyx mori* larvae infected with IL-5-expressing recombinant virus, to cultures of rat bone marrow cells resulted in an increase in the number of Luxol-fast-blue staining eosinophils in a time- and concentration-dependent manner. After 6 days culture with 100 pM recombinant rat IL-5, more than 90% of the bone marrow cells were eosinophil. The contents of major basic protein (MBP) in the bone marrow cells determined by Western blot analysis using a polyclonal antibody to rat MBP were also increased by recombinant rat IL-5 (100 pM). Furthermore, intravenous injections of recombinant rat IL-5 twice a day for six consecutive days increased the population of eosinophils in peripheral blood cells and in bone marrow cells. These findings indicate that rat IL-5 induces terminal differentiation and proliferation of progenitor cells to mature eosinophils in rats.

Keywords: Interleukin-5; Eosinophil; Bone marrow cell; Eosinophil differentiation factor; Major basic protein

1. Introduction

Eosinophils are suggested to play important roles in the pathogenesis of allergic inflammation such as bronchial asthma and atopic dermatitis [1–4]. In patients suffering from asthma, the number of eosinophils in bone marrow and peripheral blood and at the inflammatory site is increased [5–8]. At the inflammatory site, infiltrating eosinophils release various cytokines including interleukin-1 (IL-1), IL-4, IL-8, IL-10 and interferon-γ, regulated upon activation in normal T cells expressed and secreted (RANTES) and macrophage inflammatory protein-1α [9] and mediators such as leukotriene C_4 [10–12], platelet-activating factor [13,14] and granule proteins including major basic protein (MBP), eosinophil cationic protein, eosinophil-derived neurotoxin and eosinophil peroxidase [15–17], resulting in the tissue damage and bronchial hyperresponsiveness [12,14–17].

IL-5 is produced mainly by activated T cells and mast cells [18]. In humans, IL-5 shows various biological activities such as eosinophil differentiation factor (EDF) activity [19–22] and eosinophil survival-enhancing activity [23]. The terminal differentiation and the proliferation of progenitor cells to mature eosinophils are induced by EDF activity of IL-5 [19–22,24,25], resulting in the increase in the number

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0925-4439/00/$ – see front matter
PII: S0925-4439(00)00002-8

of eosinophils in allergic inflammation [26–28]. Recently, we have prepared recombinant rat IL-5 from the hemolymph of silkworm *Bombyx mori* (*B. mori*) larvae infected with cysteine proteinase-deleted virus containing cDNA for rat IL-5 [25] and found that recombinant rat IL-5 possesses EDF activity using the methylcellulose-culture system [24] of rat bone marrow cells [25]. However, in rat species, it has not been clarified whether the generation of rat eosinophils is induced by rat IL-5 in vivo. In the present study, we examined whether the recombinant rat IL-5 increases the number of eosinophils in liquid culture of rat bone marow cells and in vivo.

2. Materials and methods

2.1. Collection of rat bone marrow cells

Bone marrow cells were collected from thighbones and shinbones of rats (Sprague–Dawley strain, male, specific pathogen-free, weighing 200–250 g, Charles River Japan, Kanagawa, Japan) as described previouly [29]. The rats were treated in accordance with procedures approved by the Animal Ethics Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan. After 3 washes with minimum essential medium alpha (αMEM, Gibco-BRL, Gaithersburg, MD, USA), the bone marrow cells were used for the subsequent experiments.

2.2. Culture of rat bone marrow cells

Bone marrow cells were suspended at 4×10^5 cells/ml in αMEM containing 30% (v/v) fetal bovine serum (FBS, Flow Laboratories, North Rydge, NSW, Australia), 1% (w/v) bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) and the various concentrations of recombinant rat IL-5. The recombinant rat IL-5 was prepared from the hemolymph of silkworm *B. mori* larvae infected with cysteine proteinase-deleted virus containing cDNA for rat IL-5 as described previously [25]. Five milliliters of the cell suspension was poured into each well of a 6-well culture dish (Corning, Corning, NY, USA) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After incubation, the cells were harvested and the total number of cells that excluded Trypan blue dye was determined using a hemocytometer. A portion of the cells was smeared on a slide glass and stained with Luxol-fast-blue (EM Science, Cherry Hill, NJ, USA) and hematoxylin (Merck, Darmstadt, Germany) to identify eosinophils and the percentage of eosinophils was calculated. The total number of eosinophils was then determined.

2.3. Immunoblotting for MBP

Rat MBP was detected by a modification of the method described by Nittoh et al. [30]. After incubation for 3 and 6 days in the presence and absence of recombinant rat IL-5 (100 pM), bone marrow cells (1×10^5 cells) were boiled for 5 min in loading buffer (31.25 mM Tris–HCl (pH 6.8), 1% (w/v) sodium dodecylsulfate, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue), applied to a 15% (w/v) polyacrylamide gel and subjected to electrophoresis at 125 V for 2 h. After electrophoresis, proteins in the gel were transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Non-specific binding sites were blocked by incubation for 1 h at room temperature in Block Ace (Dainippon, Osaka, Japan). The membrane was then incubated for 12 h at 4°C with rabbit anti-MBP antibody [30] at a concentration of 0.1 μg/ml. After 3 washes, the membrane was incubated at 4°C for 3 h with a 1:5,000 dilution of biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). The reaction products were incubated for 30 min at room temperature with ABC reagent (Vector Laboratories) and visualized by ECL Systems (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was exposed to a Kodak X-Omat AR film (Eastman Kodak, Rochester, NY, USA) for 5 min at room temperature.

2.4. Detection of eosinophils in peripheral blood and bone marrow of rats

Rats (Sprague–Dawley strain, male, specific pathogen-free, weighing 200–250 g, Charles River Japan) received intravenous injections of the sterilized saline containing 0.01% (w/v) BSA in the presence or absence of 10 nM recombinant rat IL-5 at a dose of 10 pmol/kg at intervals of 12 h for 6 days. Control

rats received the saline solution without recombinant rat IL-5 in the same way. Bone marrow cells from thighbones and shinbones and peripheral blood were collected 12 h after the last injection of the recombinant rat IL-5. The percentage of eosinophils in bone marrow cells and in white blood cells was identified after staining with Luxol-fast-blue and hematoxylin.

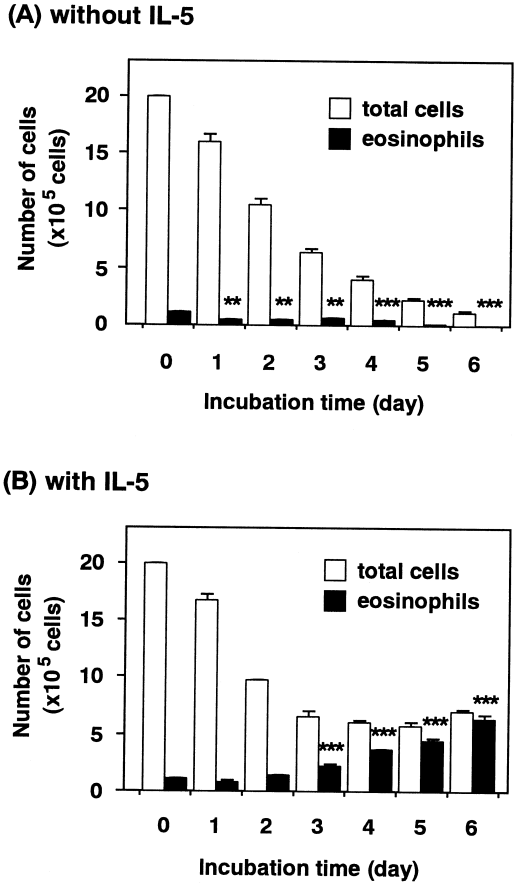

Fig. 1. Time-course of the effects of recombinant rat IL-5 on the number of eosinophils in bone marrow cells. Bone marrow cells (2×10^6 cells) were incubated at 37°C for the periods indicated in 5 ml of αMEM supplemented with 30% FBS and 1% BSA in the presence (B) or absence (A) of recombinant rat IL-5 (100 pM). After incubation, the number of total cells (open columns) was counted using a hemocytometer and the total number of eosinophils (closed columns) was calculated as follows: total number of eosinophils = (number of total cells) × (eosinophils (%)) × 0.01. Values are the means from 6 samples with S.E.M. shown by vertical bars. Statistical significance: $^{**}P<0.01$, $^{***}P<0.001$ versus number of eosinophils before incubation (day 0).

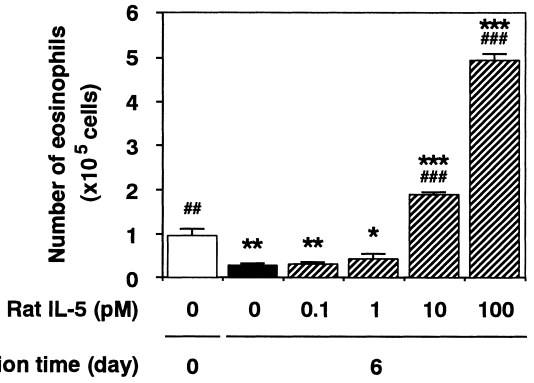

Fig. 2. Effects of recombinant rat IL-5 on the number of eosinophils in bone marrow cells. Bone marrow cells (2×10^6 cells) were incubated at 37°C for 6 days in 5 ml of αMEM supplemented with 30% FBS and 1% BSA containing the indicated concentrations of recombinant rat IL-5. After incubation for 6 days, the number of total cells was counted using a hemocytometer and the total number of eosinophils was calculated as follows: total number of eosinophils = (number of total cells) × (eosinophils (%)) × 0.01. Values are the means from 6 samples with S.E.M. shown by vertical bars. Statistical significance: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus before incubation (day 0); $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus after 6 days incubation without rat IL-5.

2.5. Statistical analysis

The statistical significance of the results was analyzed by Dunnett's test for multiple comparison.

3. Results

3.1. Time-course of the effects of recombinant rat IL-5 on the number of eosinophils in rat bone marrow cells

We examined whether the recombinant rat IL-5 increases the number of eosinophils. Before incubation, the number of eosinophils in 2.0×10^6 bone marrow cells was $(10.6\pm1.1)\times10^4$ cells (Fig. 1). When the bone marrow cells were incubated in the absence of recombinant rat IL-5, the number of total cells and eosinophils was decreased time-dependently (Fig. 1A). After 6 days incubation of the bone marrow cells in the absence of recombinant rat IL-5, the number of total cells and eosinophils was $(11.8\pm1.0)\times10^4$ and $(0.6\pm0.1)\times10^4$ cells, respectively (Fig. 1A). In contrast, the number of eosino-

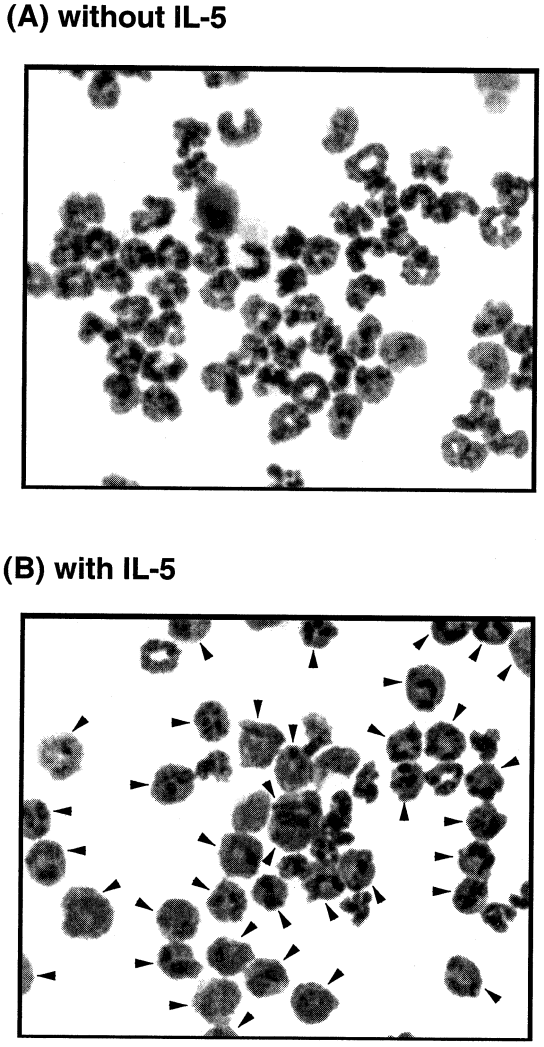

Fig. 3. Microscopic observations of the bone marrow cells. Bone marrow cells after 6 days incubation in the presence (B) or absence (A) of recombinant rat IL-5 (100 pM). The cells were smeared and stained with Luxol-fast-blue and hematoxylin. Eosinophils are shown by arrowheads. Magnification ×160.

phils increased time-dependently by 100 pM recombinant rat IL-5 (Fig. 1B). A significant increase in the number of eosinophils was observed from 3 days after incubation. In the presence of recombinant rat IL-5, the number of total cells decreased up to 3 days, but reflecting the increase in the number of eosinophils, the number of total cells did not decrease after 3 days (Fig. 1B). After 6 days incubation of the bone marrow cells in the presence of recombinant rat IL-5, the number of total cells and eosinophils was $(69.5 \pm 2.6) \times 10^4$ and $(63.9 \pm 3.9) \times 10^4$ cells, respectively. More than 90% in rat bone marrow cells were eosinophils.

3.2. *Effects of recombinant rat IL-5 on the number of eosinophils in rat bone marrow cells*

When rat bone marrow cells were incubated at 37°C for 6 days in the absence of recombinant rat IL-5, the number of eosinophils decreased compared with that before incubation (Fig. 2). In contrast, the presence of recombinant rat IL-5 increased the number of eosinophils in a concentration-dependent manner (Fig. 2). A significant increase in the number of eosinophils was observed at 10 and 100 pM recombinant rat IL-5 (Fig. 2). Microscopic observation also revealed that recombinant rat IL-5 (100 pM) increases the number of eosinophils in bone marrow cells when examined 6 days after incubation (Fig. 3). These findings indicate that recombinant rat IL-5 increases the number of eosinophils in rat bone marrow cells in vitro.

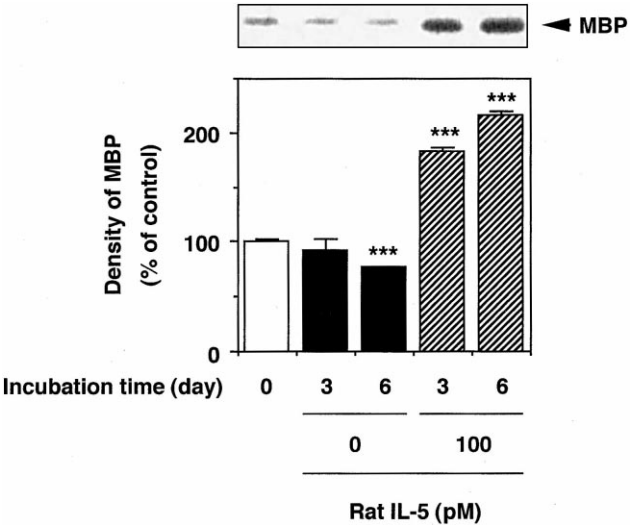

Fig. 4. Detection of MBP in cultured bone marrow cells. Bone marrow cells (2×10^6 cells) were incubated at 37°C for the periods indicated in 5 ml of αMEM supplemented with 30% FBS and 1% BSA in the presence or absence of recombinant rat IL-5 (100 pM). The cells were lysed and MBP contents in 1×10^5 cells were detected by immunoblotting (upper panel). The MBP was quantified by scanning densitometry and the density of MBP in the cells is shown in the lower panel. The mean density at day 0 is set to 100. Values are the means from 3 samples with S.E.M. shown by vertical bars. Statistical significance: ***$P < 0.001$ versus before incubation (day 0).

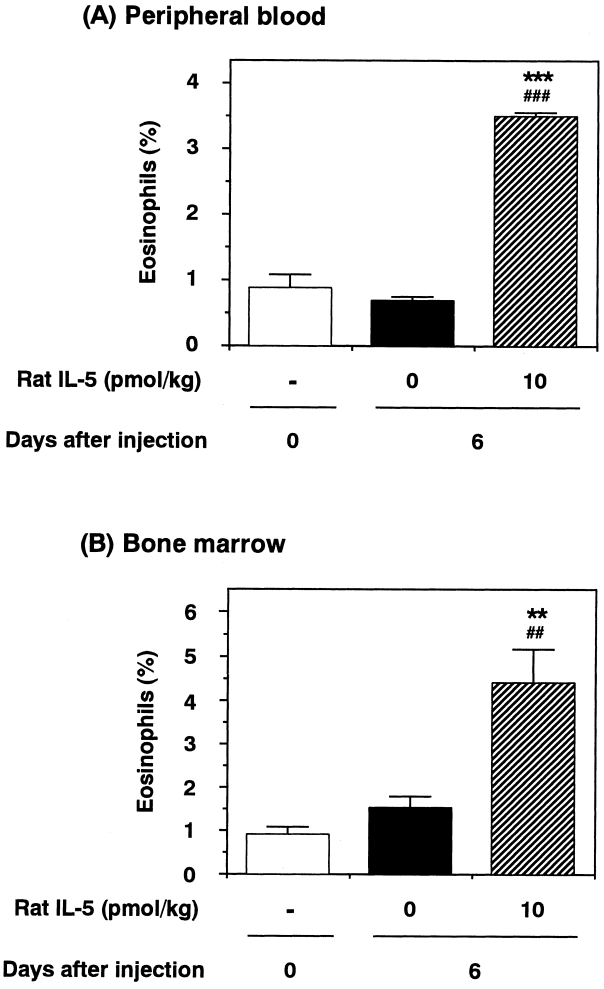

Fig. 5. Effects of recombinant rat IL-5 on the population of eosinophils in peripheral blood cells and in bone marrow cells. Recombinant rat IL-5 was injected intravenously at a dose of 10 pmol/kg at intervals of 12 h for 6 days and the number of eosinophils in peripheral blood cells (A) and in bone marrow cells (B) was counted 12 h after the last injection of recombinant rat IL-5. Percentage of eosinophils was calculated as follows: eosinophils (%) = (number of cells stained with Luxol-fast-blue/number of cells stained with hematoxylin) × 100. Values are the means from 5–6 rats with S.E.M. shown by vertical bars. Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.001$ versus before injection (day 0); $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus corresponding 6 day control.

3.3. Detection of rat MBP in cultured bone marrow cells

We analyzed whether the increase in the number of eosinophils is accompanied with the expression of MBP in bone marrow cells. When the bone marrow cells were incubated for 6 days in the absence of recombinant rat IL-5, the content of MBP in the bone marrow cells was decreased compared with that in the cells before incubation (Fig. 4). In contrast, the content of MBP was significantly increased by 100 pM recombinant rat IL-5, 3 and 6 days after incubation (Fig. 4). These findings indicate that the increase in the number of eosinophils by recombinant rat IL-5 is accompanied with the expression of MBP.

3.4. Effects of intravenous injection of recombinant rat IL-5 on the population of eosinophils in peripheral blood and bone marrow

To analyze whether the recombinant rat IL-5 induces eosinophilia, rats received intravenous injections of recombinant rat IL-5 at a dose of 10 pmol/kg at intervals of 12 h for 6 days and the percentage of eosinophils in peripheral blood cells and in bone marrow cells were determined. When rats were injected intravenously with saline solution containing no recombinant rat IL-5, the percentage of eosinophils in peripheral blood cells and in bone marrow cells did not significantly change when compared with that before injection of IL-5 (Fig. 5). In contrast, the repeated injection of recombinant rat IL-5 increased the percentage of eosinophils in peripheral blood cells and in bone marrow cells (Fig. 5). A significant increase in the percentage of eosinophils in peripheral blood cells and in bone marrow cells was also observed 3 days after injection (data not shown). These findings indicate that the repeated intravenous injection of recombinant rat IL-5 induces eosinophilia in rats.

4. Discussion

It is reported that human IL-5 and mouse IL-5 induce the generation of mature eosinophils from precursor cells in vitro in each species [19–22]. The present paper is the first to report that recombinant rat IL-5 [25] generates mature eosinophils from precursor cells in rats in vitro and in vivo.

Rats are useful animals for the analysis of allergic inflammation [25,30–34]. Nittoh et al. [30] reported that the intraperitoneal injection of *Ascaris suum*

antigen to the immunized rats increases the number of eosinophils in peripheral blood and in bone marrow. Underwood et al. [31] showed that the inhalation of the antigen ovalbumin causes the expression of IL-5 mRNA in lung tissue and the influx of eosinophils into lung tissue and airway lumen. Recently, we [25] reported that recombinant rat IL-5 induces eosinophilic colony formation in rat bone marrow cells and enhances survival of eosinophils. Therefore, in rat species, it is very possible that IL-5 contributes to eosinophilia as in human and mouse species [5–8,26–28]. To prove this possibility, we examined the effect of recombinant rat IL-5 on the generation of eosinophils in vitro and in vivo. Preliminary experiments revealed that rat neutrophils and macrophages isolated from the peritoneal cavity [35,36] are not stained with Luxol-fast-blue which specifically binds to granule proteins of human eosinophils [37]. Therefore, in this paper, we identified rat eosinophils by staining with Luxol-fast-blue. As shown in Fig. 4, the content of rat MBP determined by using the polyclonal antibody to rat MBP [30] was increased by recombinant rat IL-5, in parallel with the increase in the number of Luxol-fast-blue-positive cells in bone marrow cells (Fig. 1). Because MBP is a specific granule protein in eosinophils [38], it is strongly indicated that the Luxol-fast-blue-positive cells in rats are eosinophils and recombinant rat IL-5 induces generation of eosinophils from progenitor cells in vitro.

It is reported that IL-5 and granulocyte colony-stimulating factor (G-CSF) differentiate and proliferate the stem cells to eosinophils in humans and in mice [20,24]. Ema et al. [39] reported that, in humans, IL-3 and G-CSF differentiate $CD33^+$ $CD34^+$ stem cells to $CD33^+$ $CD34^-$ stem cells, while IL-5 differentiates and proliferates only $CD33^+$ $CD34^-$ stem cells to mature eosinophils. These reports suggest that IL-5 supports the late-stage differentiation of eosinophils. In the present study, we observed that the number of eosinophils in the bone marrow cells was increased by recombinant rat IL-5 (Figs. 1–3). Therefore, it is suggested that $CD33^+$ $CD34^-$ stem cells in rat bone marrow cells might have responded to the recombinant rat IL-5. Yamaguchi et al. [40] reported that the expression of MBP gene in human eosinophils is regulated by several transcription factors including GATA1, GATA2, C/EBPα and C/EBPβ. In C/EBPα-deficient mice, the development of eosinophils and the expression of G-CSF receptors are impaired [41]. Müller et al. [42] also showed that NF-M, a chicken homolog of C/EBPβ, induces eosinophil differentiation in human hematopoietic progenitor cells. The stimulation of eosinophils by IL-5, GM-CSF or LTB_4 activates the JAK/STAT pathway, the p44/42 MAP kinases pathway or the PI 3-kinase/Akt pathway, but the activation of p44/42 MAP kinases is not related to the eosinophil survival, aggregation, arachidonic acid release and H_2O_2 generation ([43,44], Ishihara et al., submitted for publication). However, transcription factor C/EBPβ is activated through the p44/42 MAP kinase pathway in NIH-3T3 cells [45]. Therefore, it is possible that the activation of p44/42 MAP kinase is essential for the IL-5-induced differentiation of progenitor cells to eosinophils. Further studies are required to clarify in detail the signal transduction pathway leading to differentiation and proliferation of progenitor cells to eosinophils by IL-5.

Acknowledgements

The present study was supported in part by a Grant-in-Aid for Scientific Research (B) (11470481) from the Ministry of Education, Science, Sports and Culture of Japan.

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